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A high-throughput genetic system for assessing the inhibition of proteins: identification of antibiotic resistance and virulence targets and their cognate inhibitors in *Salmonella*

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Abstract

This study describes the development of a high-throughput genetic system for producing oligopeptides that can be used to identify molecular interactions leading to inhibition of specific proteins. Using a pathogenic bacteria model, we screened a library of clones expressing intracellular oligopeptides in order to identify inhibitors of proteins involved in antibiotic resistance and virulence. This method involved transforming the pathogen with an oligopeptide-encoding plasmid library, constructed using polymerase chain reaction and an oligonucleotide template designed to produce random oligopeptides composed of 2–16 amino acids, and high-throughput screening for phenotype alterations in the pathogen. A subsequent complementation phase enabled the identification of the full-length bacterial protein inhibited by the oligopeptide. Using this method we were able to identify oligopeptides that inhibit virulence and/or drug resistance in *Salmonella*, *Shigella*, and *Escherichia coli*; specific virulence and/or drug resistance proteins of *Salmonella*, *Shigella*, and *E. coli* that are sensitive to inhibition; and putative oligopeptide-binding sites on the inhibited proteins. This system is versatile and can be extended to other pathogens for analogous studies and it can be modified for used in eukaryotic models for identifying protein interactions that can be targeted for inhibition. Additionally, this system can be used for identifying protein domains involved in any biomolecular interaction.

Keywords: Plasmid; Oligopeptide; Salmonella; Invasion; Antibiotic resistance

Proteins are often useful targets for pharmacologic intervention since many disease states can be attributed to protein dysfunction or introduction of a foreign protein. For example, a mutated protein(s) underlies the condition in certain malignancies [1]. In the case of microbial infections, the presence of a microbial protein often instigates the disease process or prevents the treatment of the infection. Consequently, methods for identifying protein inhibition are in demand and important for characterizing protein-protein interactions. The yeast two-hybrid assay is a genetic system for identifying protein-protein interactions [2] that can be relevant to perturbing the function of a given protein. More recent genetic systems directly identified peptide molecules capable of interacting with and thus perturbing vital bacterial proteins [3,4]. Whereas these genetic systems identified targets involved in growth of *Staphylococcus aureus* [3] or *Escherichia coli* [4], our initial focus was to design a genetic system that can be used to identify target proteins involved in antibiotic resistance and virulence in *Salmonella*. Like the previous studies, we chose oligopeptides, short peptide molecules comprising 2–50 amino acids, as inhibitors of the targeted proteins. Oligopeptides can serve as inhibitors of bacterial growth/virulence considering that certain bacteriocins are oligopeptides used by bacteria for the purpose of disabling neighboring bacteria [5,6]. While

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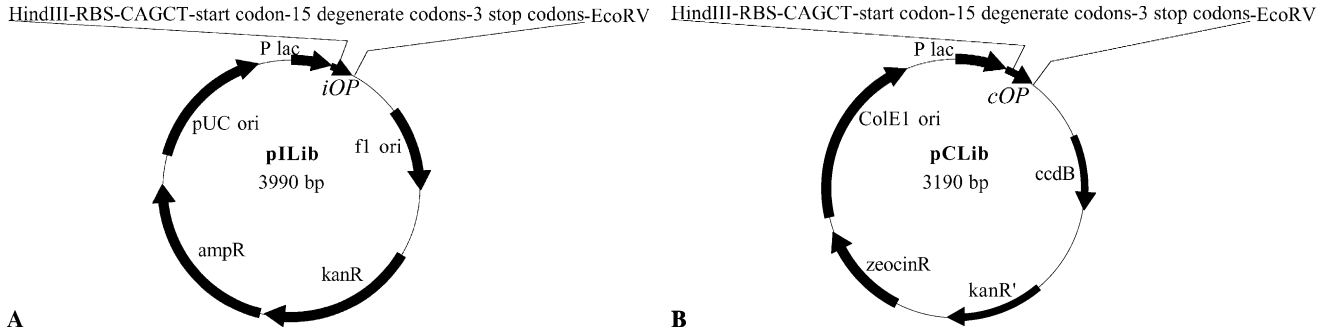

Fig. 1. Physical maps of the plasmid libraries designated as pILib and pCLib. (A) The pILib plasmid library was created using pC2.1 (Invitrogen, Carlsbad, CA) as described in the text. Inhibitory oligopeptide (*iOP*) expression is under control of the Lac promoter (P lac) which is followed by a *Hin*dIII site, a ribosomal binding site (RBS), a start codon (ATG), 15 degenerate codons (NNN), three stop codons, and an *Eco*RV site. Antibiotic resistance genes (ampR and kanR) and origins of replication (f1 ori and pUC ori) are indicated. (B) The pCLib plasmid was created using pCRXL (Invitrogen) as described in the text. Competing oligopeptide (*cOP*) expression is under control of the lac promoter (P lac) which is followed by a *Hin*dIII site, a ribosomal binding site (RBS), a start codon (ATG), 15 degenerate codons, three stop codons, and an *Eco*RV site. This plasmid contains a Zeocin resistance gene (zeocinR), a nonfunctional kanamycin resistance gene (kanR'), a suicide gene (ccdB) that ensures cOP expression, and a ColE1 origin of replication.

bioavailability and immunogenicity issues may limit the clinical application of oligopeptides, they can be useful screening tools since large quantities can be biosynthesized and an extraordinary number of different molecules are conceivable [4], and they can serve as surrogate ligands for identifying pharmacologic agents that inhibit bacterial processes [3,7]. To use oligopeptides as tools for identifying bacterial targets that are sensitive to inhibition, we created a collection of recombinant *Salmonella* clones each potentially capable of expressing a variant oligopeptide. Individual clones were screened, in novel high-throughput assays, for changes in antibiotic resistance or virulence that were subsequently attributed to a specific oligopeptide-protein interaction. We found six oligopeptides that can inhibit *Salmonella* and we identified four *Salmonella* proteins that are sensitive to inhibition.

Materials and methods

Creation of plasmid libraries

To create the plasmid inhibitory library (pILib, Fig. 1A; all plasmid abbreviations are presented in Table 3), three oligonucleotides were designed where one oligonucleotide served as a single-stranded template in a PCR[1] for which the other two oligonucleotides served as primers. The template oligonucleotide is a 90-mer with the following sequence: 5′-AAGCTTAAGGAAACAG CTATG$(NNN)_{15}$TGATAGTAAGATATCTGATAGT AA-3′. Forward and reverse PCR primers were 5′-AAGCTTAAGGAAACAGCTATG-3′ and 5′-TTA CTATCAGATATCTTACTATCA-3′, respectively. The forward primer contains a *Hin*dIII site, incorporated for orientation purposes, and a ribosomal binding site (AGGAAA) followed by a translation initiation codon. The reverse primer contains a series of stop codons flanking an *Eco*RV site incorporated for orientation purposes. Oligonucleotides were synthesized by Genemed (South San Francisco, CA). PCR was performed in an automated thermocycler (Hybaid, Teddington, UK) with 0.2-ml tubes. Reactions were performed in 20 μl containing 300 μM dATP, dTTP, dCTP, and dGTP, 2.5 mM magnesium chloride, 4 pmol of each primer, 10 mM Tris–HCl, 50 mM KCl, 0.5 unit of AmpliTaq Gold (Perkin Elmer, Foster City, CA), and 50 ng (approximately 1 pmol or 6×10^{11} molecules) of template DNA. Thermocycling entailed 95 °C for 5 min and then 40 cycles of 95 °C for 1 min, 48 °C for 30 s and 72 °C for 30 s. PCR products were ligated into pCR2.1 (Invitrogen, Carlsbad, CA; high-copy pUC plasmid, ampicillin and kanamycin resistance genes, insert expression under the control of the Lac promoter; vector map available at www.invitrogen.com) as per the manufacturer's protocol. Ligation reactions were used for transforming chemically competent *E. coli* InvFα which were inoculated into Lennox L broth (GIBCO-BRL) containing 100 μg/ml ampicillin (Sigma) and 64 μg/ml kanamycin (Sigma). To eliminate clones containing the insert in the unwanted orientation, plasmid DNA was isolated then cut with *Hin*dIII (using the site in the 5′ end of the multiple cloning site of pCR2.1 and the site in the forward primer of the cloned PCR product) and self-ligated under dilute conditions. To further eliminate clones containing the insert in the unwanted orientation, plasmid DNA was then cut with *Eco*RV (using the site in the 3′ end of the multiple cloning site of pCR2.1 and the

[1] *Abbreviations used:* PCR, polymerase chain reaction; MIC, minimum inhibitory concentration; IPTG, isopropyl-β-D-thiogalactopyranoside; HTS, high-throughput screens.

Table 1
Summary of strains and plasmids used and relevance to the genetic system

Abbreviation	Description	Relevance	Revertant strain and cOP
pILib	Plasmid inhibitory library	Plasmid designed for inhibitory oligopeptide expression in DT104	NA
Salmonella strain LB5000	Restriction(−)/modification(+) antibiotic sensitive *Salmonella*	*Salmonella* intermediate host strain used for amplifying plasmids prior to transformation into DT104	NA
DT104	*Salmonella enterica* serotype Typhimurium phagetype DT104 strain 795 (Ref. [9]))	*Salmonella* host strain for pILib	NA
pCLib	Plasmid competitive library	Plasmid designed for competing oligopeptide expression in DT104	NA
DT104/pIL10; iOP10	DT104 transformed with plasmid No. 10 from pILib; inhibitory oligopeptide expressed by DT104/pIL10	DT104/pIL10 is hypoinvasive due to inhibition of InvH by iOP10	DT104/pIL10/ pCL10;cOP10
DT104/pIL22; iOP22	DT104 transformed with plasmid No. 22 from pILib; inhibitory oligopeptide expressed by DT104/pIL22	DT104/pIL22 has diminished chloramphenicol resistance due to inhibition of FloR by iOP22	DT104/pIL22/ pCL22;cOP22
DT104/pIL153; iOP153	DT104 transformed with plasmid No. 153 from pILib; inhibitory ligopeptide expressed by DT104/pIL153	DT104/pIL153 is hypoinvasive due to inhibition of OmpC by iOP153	DT104/pIL153/pCL153; cOP153
DT104/pIL167; iOP167	DT104 transformed with plasmid No. 167 from pILib; inhibitory oligopeptide expressed by DT104/pIL167	DT104/pIL153 is hypoinvasive due to inhibition of SipA by iOP167	DT104/pIL167/ pCL167;cOP167
DT104/pIL665; iOP665	DT104 transformed with plasmid No. 665 from pILib; inhibitory oligopeptide expressed by DT104/pIL665	DT104/pIL665 has diminished ceftriaxone resistance due to expression of iOP665	DT104/pIL665/ pCL665;cOP665
202/37/pCL342; cOP342	*Salmonella* strain 202/37 (Ref. [23]) transformed with pCL342	202/37/pCL342 has diminished amikacin resistance due to cOP342	NA
pBT, pTRG	Plasmids used in two-hybrid analyses	Demonstration of iOP and full-length protein interactions	NA
DT104 ASSuT	Wild-type DT104 with a truncated *floR* gene	Used for determining background chloramphenicol resistance in DT104	NA
DT104/pAS-floR	DT104 transformed with a plasmid encoding for an antisense *floR* transcript	Used for comparing the effect of direct physical inhibition of FloR versus inhibition of FloR expression	NA
E. coli K99CVM934/pIL22; *Klebsiella pneumoniae*/pfloR(Δkan)/pIL22(Δamp); LB5000/pfloR(Δkan)/pIL22(Δamp)	*E. coli* K99 CVM934 (Ref. [22]) transformed with pIL22; *Klebsiella pneumoniae* or *Salmonella* strain LB5000 cotransformed with plasmids encoding FloR and iOP22	Demonstrates the effect of iOP22 on other bacteria expressing FloR	*E. coli* K99 CVM934/ pIL22/pCL22;cOP22
Klebsiella pneumoniae strain MCV37	A florfenicol-sensitive, chloramphenicol-resistant strain that expresses a chloramphenicol-modifying enzyme	Demonstrates the specificity of iOP22-mediated inhibition of FloR	NA
Shigella flexneri/plL153	*Shigella flexneri* transformed with pIL153	Demonstrates the effect of iOP153 on other bacteria expressing OmpC	*Shigella*/pIL153/ pCL153;cOP153
E. coli InvFα/invasin/pIL153(Δamp)	*E. coli* cotransformed with plasmids encoding invasin and iOP153	Demonstrates the specificity of iOP153-OmpC interaction	NA

site in the reverse primer of the cloned PCR product) and self-ligated under dilute conditions. Ligation reactions were used for electrotransforming restriction(−)/modification(+) antibiotic-sensitive *Salmonella* strain LB5000 (all strain abbreviations are presented in Table 1) in order to maintain plasmid fidelity in wild-type *Salmonella* [8]. Plasmids were then amplified, isolated, and electrotransformed into *Salmonella enterica* serotype Typhimurium phagetype DT104 (DT104) strain 795 (ampicillin, ceftriaxone, and chloramphenicol/florfenicol resistant; [9]). DT104 strain 795 was chosen due to its relatively elevated minimum inhibitory concentration (MIC) for chloramphenicol (128 μg/ml), its resistance to ceftriaxone that could be utilized in a parallel inhibition of ceftriaxone resistance assay, and its susceptibility to kanamycin that could be exploited for selection of transformants.

To create the plasmid competitive library (pCLib, Fig. 1B), *Hin*dIII/*Eco*RV fragments from pILib were excised and ligated into pCRXL (Invitrogen) that had the kanamycin resistance cassette previously eliminated by restriction digestion and self-ligation. Transformants were selected using 25 μg/ml Zeocin (Invitrogen). Plasmids were amplified and isolated from *E. coli* InvFα then electrotransformed into *Salmonella* strain LB5000. Plasmids were then amplified, isolated, and electrotransformed into electrocompetent DT104/pIL10, DT104/pIL22, DT104/pIL153, DT104/pIL167, or DT104/pIL665.

Screening for an inhibition of antibiotic resistance

Individual DT104/pILib colonies were grown in separate wells of 96-well microtiter plates containing 64 μg/ml kanamycin (for maintenance of pILib). Approximately 10^5 CFU were transferred into individual wells of 96-well microtiter plates containing the test antibiotic (32 μg/ml chloramphenicol, 64 μg/ml ceftriaxone, or 64 μg/ml amikacin), 64 μg/ml kanamycin, and 2 mM IPTG (for induction of oligopeptide expression). Cultures were then incubated overnight at 37 °C and growth was assessed in each well. DT104/pILib clones with diminished growth were recovered and used in subsequent studies in which pILib-mediated changes in MIC values were assessed in triplicate in three separate assays (i.e., $n = 9$). MIC values were determined by inoculating 10^6 bacteria into 1-ml aliquots of Mueller–Hinton broth (DIFCO) containing twofold serial dilutions of antibiotics as per the National Committee on Clinical Laboratory Standards guidelines [10]. Bacteria were grown aerobically and MIC values were based on the lowest concentration of antibiotic that inhibited growth. As a control, bacteria were grown in high-glucose media (SOC, Invitrogen) in order to minimize oligopeptide expression from the Lac promoter.

Creation of chloramphenicol/florfenicol-resistant and susceptible strains

DT104 is chloramphenicol/florfenicol-resistant due to the presence of *floR* [11], a gene encoding a chloramphenicol/florfenicol efflux protein [12]. Chloramphenicol/florfenicol-resistant *Salmonella* strain LB5000 and *Klebsiella pneumoniae* (antibiotic-sensitive wild type) were created by transforming these strains with pfloR(Δkan), a plasmid bearing the *floR* gene [13] that had the kanamycin resistance cassette eliminated by restriction digestion and self-ligation, and selecting in ampicillin (resistance conferred by pfloR(Δkan)) and chloramphenicol. These strains were subsequently transformed with pIL22(Δamp), i.e., pIL22 with the ampicillin resistance cassette eliminated by restriction digestion and self-ligation. LB5000/pfloR(Δkan)/pIL22(Δamp) and *K. pneumoniae*/pfloR(Δkan)/pIL22(Δamp) were selected in ampicillin and kanamycin and then evaluated for changes, relative to LB5000/pfloR(Δkan) and *K. pneumoniae*/pfloR(Δkan), in chloramphenicol resistance. For control studies, chloramphenicol/florfenicol resistance was assessed in DT104 containing an antisense *floR* transcript or a deletion in the 3′ end of native *floR* [14].

Screening for an inhibition of invasion

Single DT104/pILib colonies were grown in individual wells of 96-well microtiter plates containing 64 μg/ml kanamycin and 2 mM IPTG in Lennox L broth. Approximately 10^6 CFU of DT104/pILib was then inoculated into individual wells of 96-well microtiter plates containing 10^4 HEp-2 cells (i.e., multiplicity of infection equal to 100). Cultures and cells were subjected to a gentamicin protection assay [15]. In this assay, bacteria invading tissue culture cells are protected from extracellular gentamicin. Following eukaryotic cell lysis that liberates intracellular bacteria, bacterial culture medium was added and plates were shaken for 1 h at 37 °C and then grown statically overnight at 30 °C. This protocol was determined to be adequate for visually identifying *Salmonella* with impaired invasiveness, i.e., by visually inspecting for a dramatic decrease in bacterial culture density relative to control wells. Bacteria with apparent changes in invasion were recovered and used in subsequent studies to quantitate the changes in invasion by enumerating bacteria recovered from tissue culture cells. Assays were performed in triplicate. As a control, bacteria were grown in SOC in order to minimize inhibitory oligopeptide (iOP; all oligopeptide abbreviations are presented in Table 1) expression from the Lac promoter. For comparison, similar assays were performed for antibiotic-sensitive *Shigella flexneri*, antibiotic-sensitive enteropathogenic *E. coli*, and an *E. coli* transformed with a plasmid containing the invasin gene from *Yersinia* [16] as described recently [17].

Return-of-function studies

Approximately 5×10^{11} DT104/pIL/pCLib cotransformants were pooled. For antibiotic resistance and DT104/pIL22/pCLib or DT104/pIL665/pCLib, subpools containing 10^6 bacteria were assessed for the ability to survive in 96-well microtiter plates with each well containing the test antibiotic (32 μg/ml chloramphenicol or 64 μg/ml ceftriaxone), 64 μg/ml kanamycin (for maintenance of pILib), and 25 μg/ml Zeocin (for maintenance of pCLib). Bacteria from wells with visible growth were plated on media containing antibiotics and individual clones were subjected to MIC determinations and plasmid DNA sequencing. For DT104/pIL153/pCLib, DT104/pIL10/pCLib, DT104/pIL167/pCLib, and invasion, subpools containing 10^6 bacteria were assessed for the ability to invade HEp-2 cells in 96-well microtiter plates. Bacteria from wells with visible growth were plated on media containing antibiotics and individual clones were selected, in a manner similar to that done for the identification of invasin from *Yersinia* [16], for quantitative invasion assays.

Bacterial two-hybrid analysis

Plasmid inserts were amplified from pIL10, pIL22, pIL153, pIL167, or pIL665 using PCR and then ligated into pBT as per the manufacturer's (Stratagene) protocol. DNA sequences encoding FloR, OmpC, SipA, and InvH were amplified from DT104 DNA using PCR and then ligated into pTRG as per the manufacturer's (Stratagene) protocol. Two-hybrid screening was performed using the BacterioMatch system (Stratagene) using the following cotransformations: pBT/IL10+pTRG/InvH, pBT/IL22+pTRG/FloR, pBT/IL153+pTRG/OmpC, pBT/IL167+pTRG/SipA, and pBT/IL665+pTRG/CL665. Cotransformants displayed the carbenicillin/kanamycin resistance phenotype that represents bait protein (iOP10, 22, 153, 167, or 665)-prey protein (*Salmonella* protein) interactions. No interaction was observed for pBT/IL22+pTRG/OmpC or pBT/IL153+pTRG/FloR cotransformants (data not shown).

Results

Outline of the system

As depicted in Fig. 2, a plasmid-based system was utilized to identify oligopeptides that can inhibit an assessed phenotype and to subsequently identify the protein inhibited by the oligopeptide. We engineered a plasmid library (pILib, Fig. 1A) designed to encode for all possible oligopeptides ranging from 2 to 16 amino acids in length. That is, the library theoretically encodes for 4×10^{19} different oligopeptides. An unknown subset of oligopeptides was biosynthesized by wild-type DT104 transformed with pILib. DT104 was chosen since individual clinical isolates can be resistant to as many as 13 different antibiotics [18] and since recent studies indicate

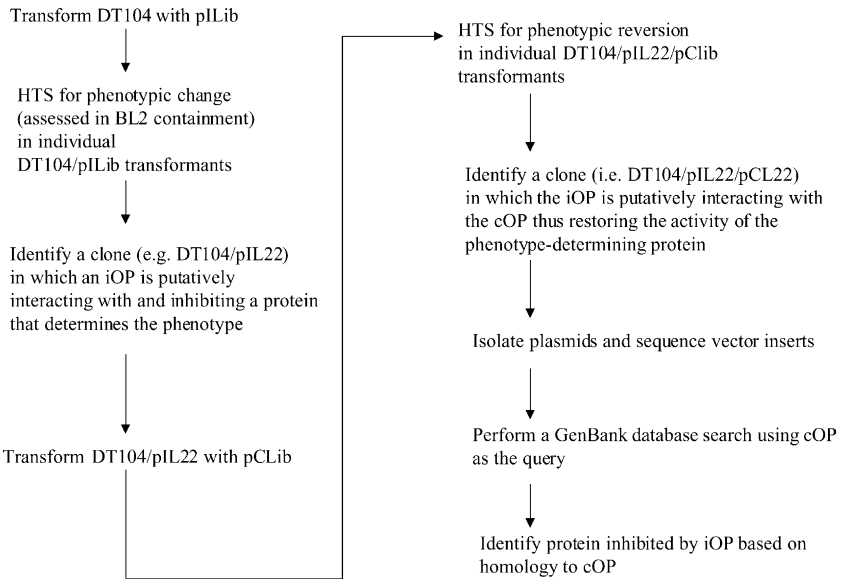

Fig. 2. Schematic of the plasmid-based oligopeptide inhibition/competition system. DT104 is transformed with pILib and phenotype alterations are assayed using high-throughput screens (HTS) and Biosafety Level 2 containment. Single pIL transformants, in which an inhibitory oligopeptide is interacting with and thus inhibiting an unknown protein, are isolated and subjected to secondary transformation. As shown in the upper right corner, pIL/pCLib clones are assayed in the same phenotype HTS until a revertant is isolated. Reversion is dependent upon sequestration of the inhibitory oligopeptide by the competing oligopeptide that restores activity to the unknown protein. Plasmids are then isolated from the revertant and the competing and inhibitory oligopeptides are identified. The identity of the unknown target protein is determined by performing a GenBank database search using the competing oligopeptide as the query.

that some DT104 may display a hyperinvasive phenotype [19]. Using high-throughput screens (HTS), individual DT104/pILib clones are screened for alterations in a given phenotype in which an unknown full-length DT104 protein is inhibited (Fig. 2). DT104/pILib clones with altered phenotypes are subsequently transformed with a second plasmid library, i.e., pCLib (Fig. 1B), and the HTS is repeated. DT104/pIL/pCLib clones with a revertant phenotype are then amplified for plasmid isolation. Plasmid inserts are then sequenced in order to identify iOP, i.e., the oligopeptide that perturbed the phenotype, and the competing oligopeptide (cOP), i.e., the oligopeptide that restored the phenotype. The sequence of the cOP is then subjected to a GenBank search in order to identify ''short nearly-exact'' homologies with full-length proteins.

HTS for increased chloramphenicol sensitivity

DT104/pILib transformants were used in assays that evaluated changes in chloramphenicol resistance. Chloramphenicol was chosen as the first test antibiotic since resistance is pervasive in DT104 [18] and since chloramphenicol is used in human medicine while florfenicol, a derivative of chloramphenicol [20], is used in veterinary medicine. The *floR* gene, encoding for a chloramphenicol/florfenicol efflux protein [12], confers chloramphenicol/florfenicol resistance in DT104 [11]. Since other researchers have shown that antibiotic efflux pumps are sensitive to inhibition [21], FloR was deemed to be an appropriate target for these studies.

Changes in chloramphenicol resistance were assayed using a bacteriostasis assay. While screening approximately 100,000 transformants, we identified a DT104/pLib clone (DT104 strain 795 [9] transformed with a plasmid hence designated as pIL22) with an increased sensitivity to chloramphenicol (Fig. 3). Intracellular expression of iOP22, the oligopeptide encoded by pIL22, reduced the MIC of chloramphenicol from 128 to 32 μg/ml. This reduction was observed in three separate independent assays in which each strain was assayed in triplicate. The iOP22-mediated effect on chloramphenicol resistance was not as dramatic as that observed when *floR* was truncated in DT104 (DT104 ASSuT), although

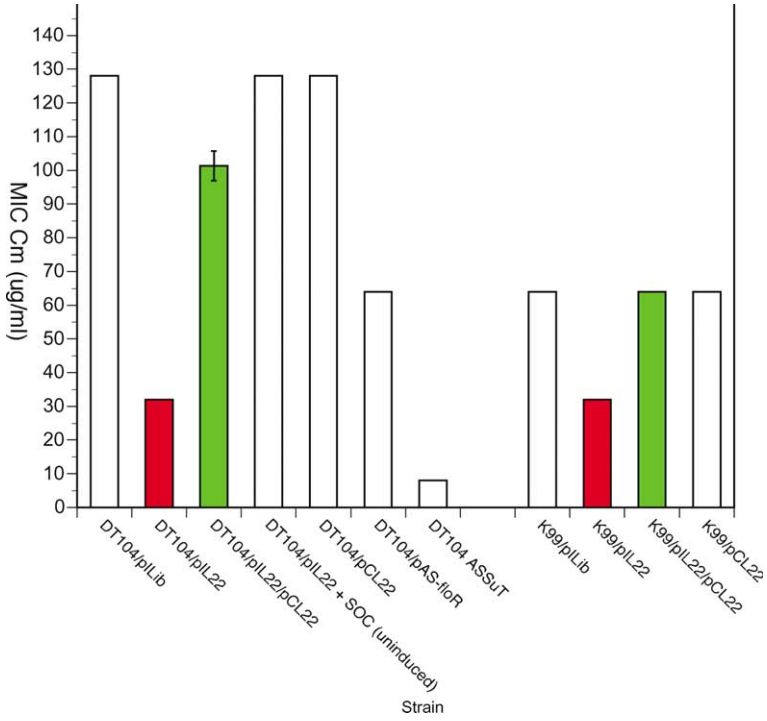

Fig. 3. Changes in chloramphenicol MIC (MIC Cm) values in relationship to transformation with pIL22. Red bars indicate an inhibition of the phenotype while green bars represent a return-of-function phenotype. Values for bacterial pools transformed with pILib were indistinguishable from nontransformed strains (data not shown). Antibiotic resistance assays were performed in triplicate and are described in the text. *Salmonella* proteins that correspond to cOPs encoded by the restorative pCLib plasmids are presented in Table 2. Strain designations are DT104, *Salmonella enterica* serotype Typhimurium phagetype DT104 strain 795 [9]; K99, *E. coli* K99 CVM934 [22]; DT104 ASSuT, a DT104 isolate with a truncated *floR* gene [14]. Transformation and treatment designations are pILib, transformation with the whole pILib library; pIL22, transformation with pIL22; pIL22/pCL22, cotransformation with pIL22 and the cOP-encoding plasmid that restored chloramphenicol resistance; pIL22 + SOC, transformation with pIL22 and growth in the presence of SOC; pCL22, transformation of only the cOP-encoding plasmid that can restore chloramphenicol resistance when cotransformed with pIL22; pAS-floR, transformation with a plasmid encoding an antisense *floR* transcript. In most assays the chloramphenicol MIC for DT104/pIL22/pCL22 was determined to be 96 μg/ml since this strain displayed intermediate resistance to 128 μg/ml. The lack of an error bar indicates an SE value equal to zero.

Table 2
Specificity of the interactions that led to an inhibition of antibiotic resistance or invasion in *Salmonella*

Strain	Plasmid used in transformation	Parameter assessed	% decrease following transformation[a]
DT104	pIL22	Chloramphenicol MIC	75 ± 0
DT104	pIL22	Florfenicol MIC	50 ± 0
Salmonella strain LB5000/pfloR(Δkan)	pIL22(Δamp)[b]	Chloramphenicol MIC	50 ± 0
E. coli K99 CVM934 (Ref. [22])	pIL22	Chloramphenicol MIC	50 ± 0
Klebsiella pneumoniae/pfloR(Δkan)	pIL22(Δamp)[b]	Chloramphenicol MIC	50 ± 0
Salmonella strain 202/37 (Ref. [23])	pIL22[c]	Chloramphenicol MIC	0
Klebsiella pneumoniae strain MCV37	pIL22	Chloramphenicol MIC	0
DT104	pIL153	Invasion	83 ± 15
Shigella flexneri	pIL153	Invasion	45 ± 7
enteropathogenic *E. coli* (Ref. [25])	pIL153	Invasion	12 ± 2
E. coli/invasin (Refs. [16,17])	pIL153(Δamp)[d]	Invasion	6 ± 1

Transformations and phenotype assays were performed as described in the text.

[a] Relative to pretransformation values. Assays performed in triplicate.

[b] pIL22 with the ampicillin resistance gene removed for cotransformation purposes; i.e., both pfloR and pIL22 possess ampicillin and kanamycin resistance genes, although pfloR(Δkan)does not confer kanamycin resistance.

[c] The iOP-encoding sequence was transferred from pIL22 into pCRXL due to the kanamycin resistance of *Salmonella* strain 202/37 (see footnote in Table 3).

[d] The ampicillin resistance gene was deleted from pIL153 since the plasmid encoding invasin also confers ampicillin resistance.

the effect exceeded that of antisense-mediated inhibition of *floR* expression in DT104 (DT104/pAS-floR). Chloramphenicol resistance was not altered when DT104/pIL22 was grown in the presence of SOC, i.e., under repressed conditions that minimize iOP expression. The pIL22-mediated perturbation of chloramphenicol resistance was also observed for florfenicol resistance (Table 2). Inhibition of chloramphenicol resistance was also observed in wild-type *E. coli* K99 CVM934 (chloramphenicol/florfenicol resistant; [22]) transformed with pIL22 (Fig. 3), *S. enterica* serotype Typhimurium strain LB5000 cotransformed with pfloR(Δkan) and pIL22(Δamp), and *K. pneumoniae* cotransformed with pfloR(Δkan) and pIL22(Δamp) (Table 2). However, inhibition of native chloramphenicol resistance was not observed in *K. pneumoniae* ATCC MCV37 (chloramphenicol and ceftriaxone resistant) or *S. enterica* serotype Typhimurium strain 202/37 (kanamycin and amikacin resistant; [23]) (Table 2).

HTS for increased ceftriaxone and amikacin sensitivity

Parallel screening of DT104/pILib transformants led to the identification of an iOP for ceftriaxone resistance (iOP665) and an iOP for amikacin resistance (iOP342) as shown in Table 3. The amikacin resistance studies were performed using *S. enterica* serotype Typhimurium strain 202/37, an amikacin-resistant strain that expresses a specific aminoglycoside acetyltransferase [23]. The effects of iOP665 and iOP342 were not as pronounced as that observed for iOP22.

HTS for an inhibition of invasion

To screen the library for potential inhibitors of invasion, DT104/pILib transformants were evaluated for changes in the ability to invade host cells. Host cell invasion is an accurate predictor of virulence and many bacteria utilize cellular invasion as a method for causing disease [24]. Changes in invasiveness were evaluated using a tissue culture gentamicin protection assay [15]. As shown in Fig. 4, we identified a DT104/pILib clone (DT104 strain 795 transformed with a plasmid hence designated as pIL153) that caused a 5-fold decrease in the invasion of DT104. This clone was identified after screening approximately 8000 transformants. Transformation of pIL153 did not have any direct effects on DT104 viability or gentamicin resistance (data not shown). Invasion was not altered when DT104/pIL153 was grown in the presence of SOC. An inhibition of invasion was also observed in *S. flexneri* transformed with pIL153 (Fig. 3) but not in enteropathogenic *E. coli* [25] transformed with pIL153 or *E. coli* InvFα cotransformed with a plasmid containing the invasin gene from *Yersinia enterocolitica* [16] and pIL153(Δamp) (Table 2). Further studies enabled the identification of two other DT104 transformants, DT104/pIL10 and DT104/pIL167, with impaired invasiveness (Table 3).

HTS for return-of-function for either antibiotic resistance or invasion

In order to identify the bacterial proteins inhibited in DT104 transformants with altered phenotypes, we transformed these clones with a second oligopeptide plasmid library and screened for a return-of-function phenotype. The second library is designated as pCLib and is depicted in Fig. 1B. Specifically, cOPs expressed from pCLib represent competitive inhibitors of an iOP; i.e., pCLib encodes for cOPs (“minipeptides” [26]) that may represent potential binding sites on the full-length bacterial proteins targeted by the iOPs. Minipeptides/cOPs were deemed as more suitable competitors than

Table 3
Summary of the interactions that led to an inhibition of antibiotic resistance or invasion in *salmonella*

Strain	Parameter assessed	% decrease[a]	Frequency[b]	Amino acid sequence of iOP	Amino acid sequence of cOP and corresponding homologous region of the protein inhibited by the iOP[c]	*Salmonella* protein putatively inhibited by the iOP	Footnotes
DT104/pIL22	Chloramphenicol resistance	75	1×10^{-5}	MDLSWLVRAGY	MKTRRSVLPIFASPAF VKTRRSTLPLFASPAF	FloR	cOP22 is homologous to amino acids 199–214 of FloR (GenBank Accession Number AF097407).
DT104/pIL665	Ceftriaxone resistance	50	5×10^{-6}	MNAVGNGPAKAMHF-GY	MSAEWGNGFVPW	Unknown	A homologue for the cOP665 sequence was not identified in a GenBank database search.
202/37/pCL342	Amikacin resistance	50	1×10^{-5}	MRHFKADGNM-VYVQPG	ND	ND	Not determined since strain 202/37 is ampicillin and kanamycin resistant and thus this strain was transformed with pCLib in order to identify the amikacin resistance inhibitor; i.e., cOP studies were not feasible and thus pCL342 encodes for an iOP.
DT104/pIL153	Invasion	80	1×10^{-4}	MPSLRQAWYRARIH	MPALYVAGAANAADI VPALLVAGAANAASI	OmpC	cOP153 is homologous to amino acids 10–24 of OmpC (Accession Number AP039309), an outer membrane protein involved in *Salmonella* adherence to host cells [27]. Adherence, evaluated as described previously [13], of strain DT104/pBL153 was 1.7 ± 0.2 bacteria/cell whereas adherences for DT104/pLib and DT104/pIL153/pCL153 and DT104/pCL153 (see Fig. 4 legend for designations) were 7.5 ± 0.4 bacteria/cell, 7.8 ± 0.9 bacteria/cell, and 7.2 ± 0.9 bacteria/cell, respectively.
DT104/pIL167	Invasion	45	2×10^{-4}	MAGFAGDDLPRGVFP	MLPEAKRAEGKLENLE WLPEAKKAEAKLENLE[i]	SipA	cOP167 is homologous to amino acids 141–156 of SipA (Accession Number AAA86618), a secreted invasion protein [30] that physically interacts with actin thus stabilizing actin polymers during invasion [29] iOP167 is homologous to amino acids 19–32 of actin (Accession Number NP_001605).
DT104/pIL10	Invasion	50	2×10^{-4}	MFYAFDMPFLH-GYDHG	MSASKRNTOSSSVFCE MSASKNNTISSSVFCE	InvH	cOPIO is homologous to amino acids 71–86 of InvH (Accession Number UB84275), a protein involved in regulating invasion [31].

Library screening, inhibitor competition, and two-hybrid analyses were performed as described in the text.
[a] Relative to pILib transformant controls. Assays performed in triplicate.
[b] Equals the inverse of the number of transformants screened as part of the evaluation process.
[c] Sequences of homologous regions of full-length targets are provided below that for the competing minipeptide.

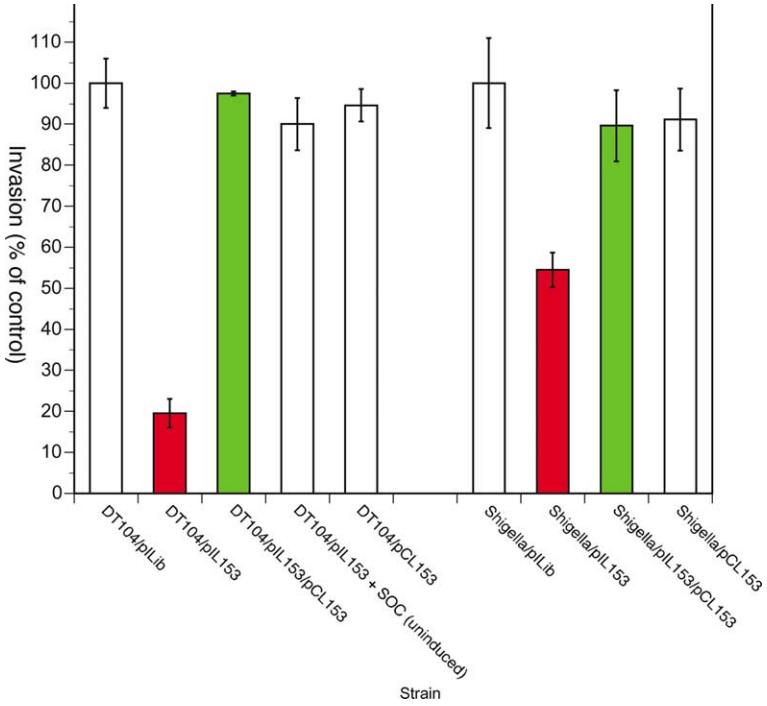

Fig. 4. Changes in tissue culture invasion in relationship to transformation with pIL153. Red bars indicate an inhibition of the phenotype while green bars represent a return-of-function phenotype. Values for bacterial pools transformed with pILib were indistinguishable from nontransformed strains (data not shown). Invasion assays were performed in triplicate and are described in the text. Strain designations are DT104, *Salmonella enterica* serotype Typhimurium phagetype DT104 strain 795(9); Shigella, *Shigella flexneri.* Transformation and treatment designations are pILib, transformation with the whole pILib library; pIL153, transformation with pIL153; pIL153/pCL153, cotransformation with pIL153 and the cOP-encoding plasmid that restored invasion; pIL153 + SOC, transformation with pIL153 and growth in the presence of SOC; pCL153, transformation of only the cOP-encoding plasmid that can restore invasion when cotransformed with pIL153. Raw percentage invasion values (mean ± SE of triplicates) are as follows: DT104/pILib (i.e., control), 11.8 ± 0.5%; DT104/pIL153, 2.3 ± 0.4%; DT104/pIL153/pCL153, 11.5 ± 0.1%; DT104/pIL153 + SOC, 10.6 ± 0.6%; DT104/pCL153, 11.2 ± 0.1%; *Shigella*/pILib (i.e., control), 7.7 ± 0.6%; *Shigella*/pIL153, 4.2 ± 0.2%; *Shigella*/pIL153/pCL153, 6.9 ± 0.4%; *Shigella*/pCL153, 7 ± 0.2%. Percentage invasion equals 100(number of bacteria added/number of bacteria recovered).

full-length proteins since they can competitively sequester the iOP, via oligopeptide-oligopeptide (iOP-cOP) interactions that prevent the iOP-protein interactions, without independently activating biosignals [26].

A competition between the iOP expressed from pIL22 (iOP22), which inhibited chloramphenicol resistance, and a cOP homologous to a part of FloR was identified using this system (Fig. 3 and Table 3). That is, the inhibition of chloramphenicol resistance occurs as a result of an iOP22-FloR interaction that inhibits FloR as indicated in Fig. 5. Expression of cOP22, in the absence of pIL22, had no effect on the native chloramphenicol resistance of DT104 (Fig. 3). The iOP22-FloR interaction was also observed in a two-hybrid system (not shown) and in other bacteria expressing FloR (Fig. 3 and Table 2).

The target for ceftriaxone resistance was not identified, although we were able to identify a cOP that appears to antagonize the iOP for ceftriaxone resistance. For amikacin resistance, we were not able to pursue the competition studies since the *Salmonella* strain utilized is kanamycin resistant (see footnote in Table 3).

For invasion and iOP153, a cOP was identified using the return-of-function aspect of the system. This cOP is homologous to a part of OmpC (Fig. 4 and Table 3), an outer membrane protein involved in *Salmonella* adherence to host cells [27]. The iOP153-OmpC interaction appears to perturb invasion by disrupting eukaryotic cell adherence since we found that strain DT104/pIL153 was deficient in its ability to adhere to tissue culture cells (footnote in Table 3). Expression of cOP153, in the absence of pIL153, had no effect on the native invasion of DT104 (Fig. 3). The iOP153-OmpC interaction was also observed in a two-hybrid system (not shown) and in *S. flexneri* (Fig. 4 and Table 2) which also expresses OmpC. For iOP167 and iOP10, the targets were identified as a secreted invasion protein (SipA) and a regulator of invasion (InvH), respectively (Table 3).

Discussion

We have developed a novel genetic system for identifying and characterizing mechanistic insights into inhibiting antibiotic resistance and virulence in pathogenic bacteria. Using *Salmonella* as a model for a multiple antibiotic-resistant pathogen, we identified six oligopeptide

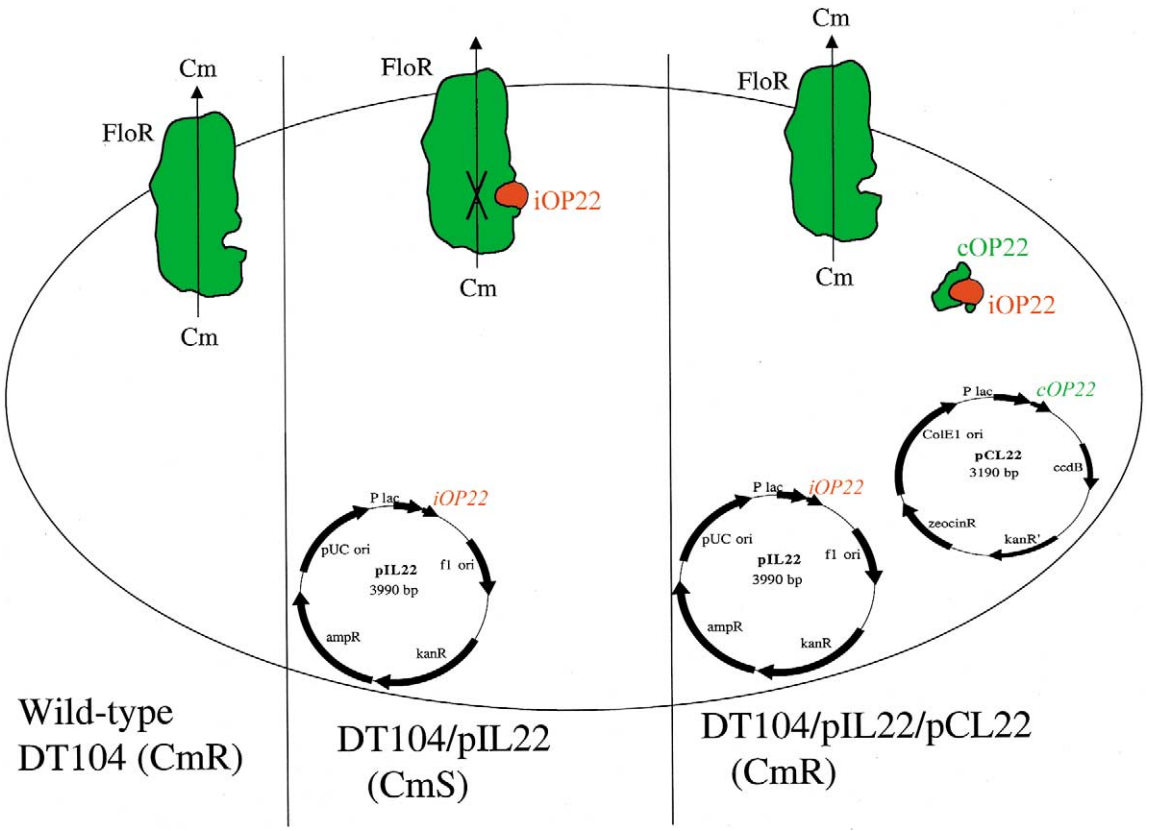

Fig. 5. Illustration of the plasmid-based oligopeptide inhibition/competition system that identified FloR as the target of the inhibitory oligopeptide (iOP22, red) expressed from pIL22. Wild-type DT104 (left frame) is chloramphenicol resistant (CmR) via expression of FloR (green), a protein that exports chloramphenicol (Cm). DT104 was transformed with pILib and chloramphenicol-sensitive (CmS) transformants were sought. A single CmS clone (DT104/pIL22, middle frame) was isolated. DT104/pIL22 was then transformed with pCLib and CmR cotransformants were sought. A single CmR cotransformant (DT104/pIL22/pCL22, right frame) was isolated. Sequencing of the oligopeptide-encoding insert of pCL22 revealed that the competing oligopeptide (cOP22, green) is homologous to a portion of FloR. The iOP22-FloR interaction appears to block the expulsion of chloramphenicol (middle frame) while the iOP22-cOP22 interaction (right frame) appears to prevent the iOP22-FloR interaction. Two-hybrid analysis confirmed that iOP22 can interact with FloR.

inhibitors of *Salmonella* and six *Salmonella* proteins that are sensitive to inhibition.

One of the oligopeptide inhibitors can directly interact with the chloramphenicol/florfenicol efflux protein FloR. The effects are specific for FloR since the inhibition was observed in bacteria expressing FloR, i.e., chromosomally in DT104 and *E. coli* K99 CVM934 or episomally in *K. pneumoniae*/pfloR(Δkan) and LB5000/pfloR(Δkan), but not in *K. pneumoniae* ATCC MCV37 and *Salmonella* strain 202/37 that express chloramphenicol-modifying enzymes.

Another oligopeptide inhibitor can directly interact with an outer membrane protein OmpC that participates in eukaryotic cell adherence and invasion. The interaction seems to be specific for OmpC since the inhibition of invasion was observed in another bacteria expressing and utilizing OmpC for invasion, i.e., *Shigella* [13], but not for enteropathogenic *E. coli* or *Y. enterocolitica*. This is not surprising since *Salmonella* and *Shigella* share a number of invasion genes [28] not present in enteropathogenic *E. coli* or *Y. enterocolitica.*

Besides identifying oligopeptide inhibitors and their cognate proteins, the system also identified a putative structure-function relationship. The iOP that inhibits SipA is homologous to amino acids 19–32 of actin and the cOP is homologous to amino acids 141–156 of SipA. This is interesting because actin and SipA physically interact during invasion [29], suggesting that amino acids 141–156 of SipA can interact with amino acids 19–32 of actin.

Interestingly, the frequency of identification was similar for each of the three antibiotic resistance phenotypes evaluated. It is difficult, however, to draw specific conclusions since only one oligopeptide-protein interaction was detected for each of the three antibiotic resistance phenotypes. It is also interesting that the frequency for identifying iOPs for invasion ($n = 3$) was 10-fold greater than that for antibiotic resistance. This observation likely reflects the larger number of proteins, and thus targets, involved in conferring invasion.

This system will be used for providing insights into the inhibition of other types of antibiotic resistance and

other pathogenic characteristics in *Salmonella*. Our goal is to use this system for generating a database of oligopeptide-protein interactions that are useful for perturbing bacterial physiology. This method represents a simple approach for studying the interaction between inhibitors and bacterial growth or virulence and for identifying novel targets for these inhibitors. Future studies will entail developing this system for identifying important eukaryotic protein interactions that can be inhibited. Using the competitive portion of this system, it may also be possible to identify protein domains involved in binding to lipid, carbohydrate, or any molecule involved in a physiologic process.

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